Modification of Infectious Bovine Rhinotracheitis (IBR) Virus in Tissue Culture and Development of a Vaccine. (23505)

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A virus isolated in bovine kidney tissue culture, and identified as the cause of IBR, has been described(1,2). Since the initial appearance of this disease in Colorado in 1950(3), it has been observed in almost every western state, and as far east as Ohio. All reports indicate that this condition is of major economic importance to the livestock industry (3-5). The propagation of the etiological agent in tissue culture made development of a vaccine feasible. This paper shows that rapid passages of IBR virus in bovine kidney tissue culture and subsequent selection by terminal dilutions have resulted in a virus so modified that it could be injected without production of disease, but with formation of antibodies.

Material and methods. Virus. One strain of IBR virus, Colorado I, was employed for the modification studies. This strain was isolated in bovine embryo kidney tissue culture roller tubes from respiratory tissues of 2 cows in the acute phase of IBR. *Tissue Culture Procedures.* Bovine embryo kidney tissue culture in roller tubes was prepared from trypsinized kidney cortex 8-9-months-old fetuses. The nutrient medium consisted of 0.5% lactalbumin hydrolysate, 5-10% horse serum, and Earle's basic salt solution containing 200 units of penicillin and 200 μg of streptomycin/ml. A more detailed description of preparation of these cultures has been given(2). To obtain larger quantities of virus, 5 g of bovine embryonic kidney cortex were minced to 1-2 mm size, suspended in 500 ml of the above-mentioned medium in Povitsky bottles, and shaken for 3-4 days at 35°C. These bottles were usually inoculated with 1 ml of infected tissue culture fluid containing not less than 4 logs of virus, shaken for an additional 4 days, and the fluids harvested. Regular passages of the virus were made by harvesting the infected tissue culture fluid and transferring 0.2 ml amounts to new cultures only after a complete cytopathogenic effect (CPE) occurred on the cells. Rapid passages consisted of transfers of infected tissue culture fluid to new cultures in 0.2 ml amounts per tube at 24-48 hour intervals, when approximately 25-50% of the cells exhibited a cytological change. To select the virus particles which were present in the majority at a given passage level, terminal dilutions were performed. Ten-fold dilutions of the infected tissue culture fluids were prepared in tissue culture medium without horse serum, and at least 3 kidney tissue culture tubes were inoculated with each dilution. The fluid from the culture inoculated with the highest dilution which still produced a CPE was harvested, 10-fold dilutions again prepared, and these dilutions inoculated into new tissue cultures. *Neutralization Test.* Equal amounts of serum or serum dilutions were mixed with equal amounts of virus in a final concentration of 100 to 1,000 $TCID_{50}$ (50% tissue culture infectious dose). This mixture was incubated for 2 hours at 37°C, 0.2 ml inoculated into each of 3-6 kidney tissue culture tubes, and observed for several days. The serum neutralization effect was recorded when approximately 100 $TCID_{50}$ of virus was observed in the virus control titration carried out simultaneously. *Cattle.* Cattle 4 months of age or older, regardless of breed or sex, were used for the laboratory part of this work. They were generally held in isolation for at least 2 weeks prior to inoculation, and daily temperature records as well as other observations were made to insure that the animals were normal before use. The susceptibility of the cattle to IBR was determined by absence of neutralizing antibodies in their serum. In tests for immunity, cattle were challenged intranasally with 1-2 ml of virulent (low passage tissue culture fluid) virus. The virus was sprayed into the nostrils with a syringe and needle partially pinched off at the end to give a spraying effect. Daily temperatures were recorded and

observations made for other signs of illness such as anorexia, depression, increased respiration, nasal discharge, hyperemia of the nasal mucosa, and the presence of serofibrinous exudate covering the surfaces of the nasal mucosa. While a moderate fever of slight duration alone might be suggestive of a reaction to challenge, no response was considered definite until one or more of the other signs of illness in addition to fever were also observed.

Results. 1. *Modification of Virus.* In experiments to modify the virulence of the IBR virus, a series of rapid passages was initiated. To obtain a large number of virus particles, the 7th regular passage of the agent was selected as starting material. After 21 consecutive rapid passages, 3 terminal dilutions were made with a portion of this 21st passage material. A larger amount of virus was then prepared by inoculating several tissue culture tubes with the 3rd terminal dilution virus and harvesting the tissue culture fluid after a complete CPE occurred. This virus is henceforth referred to as RP 21. Using a portion of the unpurified 21st rapid passage material, 19 additional rapid passages were made. This material was also passed through 3 terminal dilutions. Again, a pool of virus was prepared by inoculating tissue culture tubes with the 3rd terminal dilution virus (RP 40).

Inasmuch as a practical vaccine for cattle, employing a modified virus, would have to be administered parenterally, the intramuscular route was selected as a preliminary means of determining any reduction in virulence which might have occurred at any particular point during tissue culture passages. It had been noted that when a susceptible calf was inoculated intramuscularly with $10^{5.2}$ $TCID_{50}$ in 1 ml of the first regular passage of the original infectious material in tissue culture (T.C. #1), the animal responded after an incubation period of 2-3 days with a fever up to 104.8°F, increased respiration, anorexia, and depression. A similar response, with fever of 105.8°F, anorexia, and increased respiration, was observed when a calf was inoculated with 5 ml of the 15th regular passage in bovine kidney tissue culture titering $10^{6.4}$/ml. Accordingly, a susceptible calf was inoculated with 1 ml of tissue culture fluid containing 10^{8} $TCID_{50}$ of RP 21. Another calf was inoculated with 1 ml of tissue culture fluid containing $10^{6.2}$ $TCID_{50}$ of RP 40. Temperature response and other observations were made daily for 3 weeks, following which each animal was challenged intranasally with virulent virus. Serum samples were obtained at the time of inoculation, and again prior to challenge, Following inoculation with RP 21, a febrile response occurred within 24 hours, reaching 106°F on the 3rd day. No other signs of illness, such as anorexia or increased respiration, could be observed. The calf inoculated with RP 40 developed no fever or other signs of illness. No antibodies could be demonstrated in the pre-inoculation serum samples of these animals, although in each instance neutralizing antibody titer from 1:9 to 1:38 occurred when 2-3 week serum samples were tested. None of the animals showed any signs of illness when challenged intranasally with virulent IBR virus.

2. *Tests in Cattle.* To determine whether results similar to those above with RP 40 could be reproduced, several larger batches of virus were prepared either in trypsinized or minced bovine kidney tissue cultures using RP 40 as seed virus. (a) *Young Experimental Cattle.* One or more calves weighing from 350-500 lb were inoculated intramuscularly with each lot of virus prepared, using a total of 18 animals. Serum samples were taken at the time of inoculation, and again 3-4 weeks later. When the 2nd sample was taken, the animals were challenged intranasally with a virulent virus capable of producing typical IBR in 1 or more susceptible control animals inoculated simultaneously. None of the animals showed signs of IBR, all developed neutralizing antibodies, and resisted a challenge with virulent virus (Table I). (b) *Titration of Tissue Culture Virus in Cattle.* All cattle used in the above experiments were inoculated with 4 or more logs of virus, but provided no indication of the minimum $TCID_{50}$ of virus necessary to immunize an animal. To use this modified virus as a vaccine, it was necessary to determine the minimum amount of virus required to immunize an animal. Ten-fold dilutions of RP 40 virus were prepared in tissue culture media, and for each dilution

TABLE I. Response of Cattle Inoculated Intramuscularly with Modified IBR Virus (RP 40).

No. of cattle	$TCID_{50}$ inoc. (logs)	Febrile response	Other signs of illness	Antibody response Pre	Antibody response Post	Challenge response
2	4 –5	0/ 2*	0/ 2	0/ 2	2/ 2†	0/ 2
10	5.1–6	1/10‡	0/10	0/10	10/10	0/10
6	6.1–7	2/ 6‡	0/ 6	0/ 6	6/ 6	0/ 3§

* Numerator = No. of animals responding; denominator = No. of animals used.
† Antibody titer range from 1:3 to 1:19. ‡ A fever above 104°F lasting for only 1 day.
§ Only 3 of the 6 inoculated cattle were challenged.

1 calf was inoculated intramuscularly. The exact amount of virus in the 1 ml inoculum for each animal was determined by a simultaneous titration in tissue culture. It was found that an animal inoculated with $10^{4.2}$ $TCID_{50}$ of virus developed a definite antibody response and resisted challenge. However, $10^{3.2}$ $TCID_{50}$ of virus produced a very low antibody response which did not prevent entirely a reaction to challenge with virulent virus. Because of questionable immunity developed in this animal, it was estimated that $10^{3.7}$ $TCID_{50}$ of virus represented a minimum immunizing amount. Therefore, in all subsequent experiments, animals were inoculated with at least 10 times the minimum immunizing amount. (c) *Beef Cattle.* Since laboratory tests with the modified virus were conducted with cattle younger than those in which the disease ordinarily occurs, and since they were held in isolation rooms not subject to marked climatic changes, the modified virus was tested in heavy beef-type animals under simulated field conditions. To facilitate the use of the modified virus in the field, 2 lots of RP 40 virus were prepared and lyophilized in glass vials. This lyophilized vaccine proved to be quite stable, and when held at refrigerator temperature (4°C) for 8 months, no loss in titer could be demonstrated. With the co-

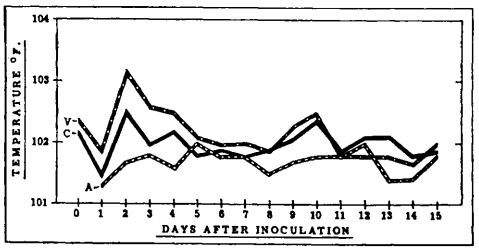

FIG. 1. Composite graph of temperature response of 30 cattle vaccinated with RP 40 virus (V), compared with temperature curves of 21 uninoculated contact controls (C) and 14 uninoculated area controls (A).

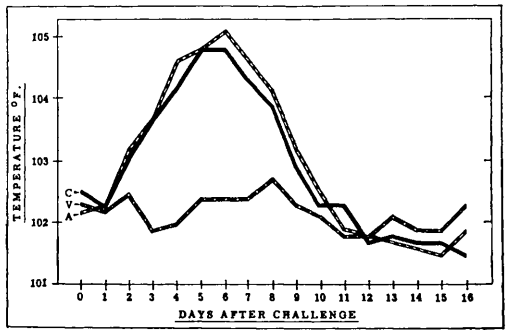

FIG. 2. Composite graph of temperature response of 30 vaccinated cattle (V), 21 contact controls (C), and 14 area controls (A) following challenge with virulent IBR virus

operation of Dr. John W. Kendrick *et al.* of the University of California School of Vet Med at Davis(6), and Dr. Rue Jensen *et al.*, School of Vet Med, Colorado State University, a total of 30 animals was used for vaccination. Each animal was inoculated intramuscularly with 2 ml of vaccine containing at least 50,000 $TCID_{50}$ of virus restored from the lyophilized state with distilled water. As controls, a total of 21 animals was held in contact with the vaccinated cattle, and 14 additional control animals were held in the same general area, but not in direct contact with them. All animals were observed daily and temperatures recorded. After 3 weeks, all the animals were challenged intranasally with virulent virus. Serum samples taken from all cattle before vaccination had no demonstrable neutralizing antibodies against IBR. The average daily temperatures for each group of cattle (Fig. 1) show that febrile response in the vaccinated group differed little from that of the controls. No signs of illness could be observed. In contrast, however, following challenge with virulent virus, the 2 control groups had a marked rise in fever although the vaccinates remained normal (Fig. 2). No signs of illness were ob-

TABLE II. Comparison between RP 40 Virus and Low Passage Virulent Virus Inoculated Intranasally into Cattle.

Inoculum	Calf No.	$TCID_{50}$ of virus inoc. × 1000	Results		
			Clinical	Antibody titer	Challenge
Modified virus (RP 40)	157	200	Normal	0	F,N,H,L,D*
	158	200	"	0	N,H,L
	159	200	"	1:3	Normal
	160	200	"	1:7	"
Virulent virus (T.C. #2)	152	320	F,H,L,N	1:23	
	153	320	F,R,D,A,H,L,N,W	1:38	
	150	3.2	F,R,D,A,H,L,N,W	Autopsied	
	155	.32	F,R,D,A,H,L,N,W	1:23	
	151	.032	Normal	0	F,N,H,L,R

* F, fever; R, increased respiration; D, depression; A, anorexia; H, nasal hyperemia; L, serofibrinous exudate; N, nasal discharge; W, loss of wt.

served in the vaccinates, except a mild fever in 1 animal, while 33 of the 35 controls developed other signs of IBR in addition to fever, and 2 died. (d) *Dairy Cattle.* Since one of the characteristic signs of illness following infection with IBR virus is abrupt cessation of milk production in the dairy animal(4,7), it was desirable to learn whether this modified virus would produce such an effect. For 5 days prior to inoculation, morning and evening temperatures and milk production were recorded for each of 5 Holstein cows. Three animals, selected at random, were inoculated intramuscularly with 2 ml of vaccine containing 2 million $TCID_{50}$ of virus. The 2 remaining animals were inoculated with a tissue culture placebo to serve as contact controls. Serum samples were taken from each animal prior to inoculation, and again 3 weeks later. For 2 weeks following inoculation, milk production and temperatures were again recorded. No change in milk production, or any other signs of illness, was noted in these animals. The virus-inoculated cattle developed antibodies, while controls remained negative.

3. *Comparison between Modified Virus and Virulent Virus by Intranasal Inoculation of Cattle.* Previous work(2,5) indicated that only by intranasal inoculation could all the signs of illness observed in the field be reproduced in experimental cattle. Therefore, this route was employed to obtain additional evidence of modification. Four susceptible animals were each inoculated intranasally with 2 ml of infected tissue culture fluid containing 200,000 $TCID_{50}$ of RP 40 virus, as determined by a simultaneous titration in tissue culture. At the same time, 10-fold serial dilutions of low tissue culture passage (T.C. #2) virulent virus of the same strain were prepared. One or more susceptible animals were inoculated intranasally with 2 ml of this material for each dilution. A simultaneous titration in tissue culture was performed. Serum samples were taken from the animals at time of inoculation, and again 3 weeks later. At the end of the 3-week period of observation, those animals showing no response to the first inoculation were challenged intranasally with virulent virus. The results of this test (Table II) show that as much as 200,000 $TCID_{50}$ of modified virus produced no signs of illness, but only 2 of the 4 animals developed demonstrable antibodies and resisted challenge. However, as little as 320 $TCID_{50}$ of virulent virus produced definite disease and antibodies. When 2 additional calves were inoculated intranasally with $10^{6.5}$ and $10^{6.8}$ $TCID_{50}$ of modified virus, respectively, mild hyperemia and a small amount of serofibrinous exudate, accompanied by a febrile response to 104.6°F for 1 day, were noted.

4. *Non-transmissibility of Virus Inoculated Intramuscularly.* In the work described above, control animals in direct contact with cattle inoculated intramuscularly with modified virus did not develop antibodies, and were susceptible when challenged. In addition, a calf held in contact with an animal inoculated in the same manner with virulent virus also remained susceptible. Since the virus apparently did not spread, deliberate attempts were made to determine whether virus could be recovered from animals inoculated by this route.

Blood and nasal washings were collected at daily intervals for 4-10 days from 5 cattle inoculated with modified virus. Specimens were also collected for as long as 27 days from 1 animal inoculated with virulent (T.C. #1) virus. The blood was defibrinated and inoculated in 0.5 ml amounts into each of several bovine kidney tissue culture tubes. After 3-5 days' incubation, the fluid was removed and new media placed in the tubes. Nasal washings were obtained by flushing the nostrils several times with tissue culture media, using approximately 20 ml of fluid. The washings were centrifuged at 5,000 rpm for 20 minutes, and the supernatant inoculated in 0.2 amounts into each of several tissue culture tubes. If yeast or mold contamination occurred in the tubes, the fluid was removed, filtered, and re-inoculated into additional tissue culture tubes. The inoculated tubes of blood and nasal washings were observed for 10 days, and if no changes in the cells occurred, they were either discarded or blind passages made. No IBR virus could be recovered from the animals tested.

Discussion. In attempts to develop a modified live virus vaccine for IBR, rapid passages were initiated to select virus variants with a high multiplication rate in bovine kidney tissue culture, assuming that such virus particles might also have a different disease-producing potential, and therefore conceivably possess less virulence. When the variant particles exceed the number of original particles, it is possible to segregate them by methods such as the terminal dilution technic.

In the data presented, it was shown that after 40 rapid passages, followed by 3 terminal dilutions, a strain of virus was recovered which was avirulent. Even in tests with a rather large group of animals inoculated intramuscularly with this strain, no response occurred except for a transient rise in temperature in some instances. This lack of response might have been due partly to the unnatural route of inoculation, since even when virulent virus was inoculated intramuscularly a febrile response, increased respiration, anorexia and depression occurred, but other signs of IBR such as nasal discharge, hyperemia, and serofibrinous exudate on the nasal mucosa were absent. Following intranasal inoculation with as little as 320 $TCID_{50}$ of virulent virus, however, all of these signs of disease could be produced. Definite evidence that RP 40 virus has been greatly modified is shown by the fact that, in contrast to virulent virus, when 625 times more RP 40 virus was sprayed into the nasal passages, not only were no signs of illness produced, but half of the animals failed to develop antibodies, indicating that this modified virus had lost much of its infectivity. It is possible that when large amounts of RP 40 virus are administered intranasally, a local reaction may occur without the development of generalized illness.

The possibility of reversion to virulence by natural back-passages always arises in connection with a modified live virus vaccine. However, it has been demonstrated that this virus did not spread from the vaccinated cattle to susceptible contacts, and could not be reisolated from nasal washings and blood of vaccinated animals, which makes this possibility very unlikely.

The inability to recover virus from intramuscularly inoculated cattle, and the fact that it requires relatively large amounts of modified virus to immunize an animal raises the question as to whether it multiplies or acts as pre-formed antigen. However, regardless of the mode of action, when 5,000 or more $TCID_{50}$ of RP 40 virus is inoculated intramuscularly, an immune response against IBR regularly occurs.

Summary. 1. A modified IBR virus was produced by rapid passages and terminal dilutions in bovine embryo kidney tissue culture. Its avirulence was demonstrated by intramuscular and intranasal inoculation of susceptible cattle. 2. Modified virus inoculated intramuscularly elicits an immune response which protects cattle against challenge with virulent virus. 3. The virus did not spread from intramuscularly-inoculated cattle to susceptible contacts, and could not be reisolated from blood or nasal washings of vaccinated cattle. 4. This modified virus prepared in tissue culture can be successfully lyophilized, thus allowing its use as a vaccine for prevention of IBR disease.

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Received July 29, 1957. P.S.E.B.M., 1957, v96.

Development of Phenylalanine Hydroxylase in Liver of the Rat.* (23506)

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Inability to convert phenylalanine to tyrosine, a reaction catalyzed by the enzyme system phenylalanine hydroxylase, is known to occur as an hereditary metabolic abnormality. Jervis(1) and Udenfriend and Bessman(2) have reported that individuals with phenylpyruvic oligophrenia almost completely lack the ability to oxidize phenylalanine, while Jervis(3) has shown that postmortem specimens of livers of two patients with this disease were inactive in *in vitro* assays for phenylalanine hydroxylase. Recently, detailed biochemical observations on liver biopsy(4) and autopsy (5) material have confirmed and extended his findings.

Since other enzymes whose deficiencies in postnatal life are associated with disorders of incomplete metabolism are known to be essentially inactive in fetal life(6)—glucose-6-phosphatase(7) and the enzymes of tyrosine oxidation(8)—it seemed worthwhile to determine if the enzyme system, phenylalanine hydroxylase, demonstrates a similar developmental pattern.

Methods. Fetal, newborn, and adult rats of the Long-Evans strain were used. Fetal animals were studied as close as possible to predicted term. They and newborn rats were killed by decapitation; adult rats by a blow to the head. Livers were removed in the cold room (temperature 2 to 4°C) and immediately placed on cracked ice. Fetal as well as newborn livers were pooled to obtain adequate samples. The livers were homogenized in the cold with a Teflon homogenizer in 2 volumes of ice-cold 0.15 M KCl. Homogenates were spun in a Spinco Model L centrifuge for 30 min. at 100,000 x gravity. The soluble fractions were assayed directly or after 3 hours of dialysis against 0.15 M KCl in 0.01 M phosphate buffer at pH 7.0. Freezing at −15°C for several days resulted in no loss of activity. The soluble fractions of livers (1.0 ml) were shaken at 35°C in air in a reaction mixture containing 2 μM of L-phenylalanine, 150 μM of phosphate buffer, pH 7.0, 5 μM of nicotinamide, and 2 μM of reduced diphosphopyridine nucleotide; final volumes were 1.75 ml. The reaction was stopped after 20 to 60 minutes incubation by addition of 1.75 ml of 10% trichloroacetic acid. Following centrifugation, formed tyrosine was measured colorimetrically(9) on suitable aliquots of the deproteinized solution. Control values obtained with reaction mixtures incubated without phenylalanine were subtracted in each case. Preparations of known activity were concurrently assayed to ensure satisfactory experimental conditions. Nitrogen was determined on aliquots of the liver soluble fractions by the Ma-Zuazaga modification of the micro-Kjeldahl method(10).

Results. Phenylalanine hydroxylase activity in dialyzed soluble fractions of livers of

* This work was supported in part by grants from Natl. Inst. of Arthritis and Meabolic Diseases, N.I.H., Assn. for Aid of Crippled Children; and Damon Runyon Memorial Fund.